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Ethylene signalling receptors and transcription factors over the grape berry development: gene expression profiling

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Summary

The ethylene signalling pathway has never been fully described in grapes. Regarded as a non-climacteric fruit, grape berry seems to ripen independently to ethylene, however 1-methylcyclopropene (1-MCP), a specific inhibitor of ethylene receptors has been shown to alter berry ripening processes. Here, we report profiles of transcript abundance of various receptors and transcription factors, associated with ethylene signalling, throughout berry development. Transcript abundance of ortholog VvETR2 gene showed a transient peak at the inception of ripening in 'Cabernet Sauvignon' berries coinciding with an internal ethylene peak, prior to colour changes. The transcripts of other orthologs such as VvRTE1 and VvEIN4 steadily increased over the berry development, while VvERS1 ortholog transcripts exhibited a peak of accumulation only when the berries were fully coloured. Finally, mRNAs of two transcription factors, VvEIN3 and VvMADS4, showed strong accumulation during the late phase of berry ripening. We also observed inflections of mRNA accumulation after incubating berry clusters with ethylene and 1-MCP (inhibitor of ethylene action). The main effect was observed with VvEIN3 transcripts that showed a significant up-regulation after incubation with 1-MCP. Furthermore, other transcript levels (VvETR2 and VvCTR1) were also increased by exogenous ethylene, once the colour change was initiated (*i.e.* 10 to 11 weeks after bloom). Some studies have already indicated that non-climacteric fruits shared signalling pathways with climacteric fruits. However, most differences between these ripening classifications remain poorly described at the genetic/molecular level. This data set will contribute to a better understanding on potential involvements of ethylene signalling in a non-climacteric fruit such as grape berry.

K e y w o r d s : grape, *Vitis vinifera,* ethylene signal, receptor, transcription factor.

Introduction

Grape has been classified as a non-climacteric fruit in the 70's and ethylene is thought to have a limited role, if any, in its ripening process (Kanellis and Roubelakis-Angelakis 1993, and references herein). However the 1-methylcyclopropene (1-MCP), an inhibitor of ethylene receptors (Blankenship and Dole 2003) was shown to affect some of the ripening changes: berry growth, acidity decrease and anthocyanin accumulation (Chervin *et al.* 2004), when applied just before the inception of ripening, a stage called veraison. At the same stage of berry development, incubating grape clusters with ethylene induced the berry enlargement and modified the transcript accumulation of several genes (Chervin *et al.* 2008).

We thought it would be worth looking at the ethylene signalling pathway in grape berry to carry out studies on the involvement of this phytohormone in grape berry development.

Nowadays, the molecular details of the early steps in ethylene signalling transduction pathway become firmly established. Genetic studies in *Arabidopsis thaliana* have provided evidence that a family of receptors mediates ethylene perception in plants (Kendrick and Chang 2008, Binder 2008). The ethylene receptor 1 (*etr1*) was the first member cloned in this family and appeared to express in different tissues. Loss-of-function mutations in ETR1 enhance sensitivity to ethylene in Arabidopsis suggesting that it functions as negative regulator in the ethylene signalling (Cancel and Larsen 2002). Besides, the loss-of-function occurs in all tissues tested suggesting a broad role of this receptor in the whole plant (Cancel and Larsen 2002). The predicted ETR1 protein has domains similar to histidine protein kinases and the receiver domain of two-component regulators (Kendrick and Chang 2008 and refs herein). So far, four other genes encoding ethylene receptors have been isolated in *Arabidopsis thaliana*: *etr2, ers1* and *ers2* (ethylene response sensors), and *ein4* (ethylene insensitive 4) reviewed previously (Kendrick and Chang 2008). Based on sequence similarities, and overall gene structure, ethylene receptors can be clustered into two subfamilies I (ETR1 and ERS1) and subfamilies II (ETR2, ERS2 and EIN4) (Guo and Ecker 2004). Furthermore, and with respect of the protein sequence, the degeneracy of the kinase domains in ETR2, ERS2 and EIN4 and the lack of response domains in ERS1 and ERS2 proteins indicate that each protein may have different role in the plant. This assumption is also supported by spatial-temporal differences in terms of mRNA expression patterns. Indeed, in *Solanum lycopersicum*, some of the five different ethylene receptors show high expression levels in green and red fruits in comparison to other plant organs (Klee 2002)

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Additionally, these receptors "sub-units" appear to be associated with another protein, called "constitutive triple response" with the corresponding gene, *ctr1*. The ethylene molecule is sensed by the ETR/ERS/EIN proteins, which activate the CTR1 complex that subsequently stops blocking the downstream cascade of the ethylene-signalling. In tomato, there are at least six different *etr* orthologs and three *ctr* orthologs (Barry and Giovannoni 2007, and refs herein). CTR1 protein complex is thought to be the key protein that unlocks the downstream signal cascade, when all receptors in its vicinity are saturated with ethylene. A new member of the receptor complex, RTE1, has been recently identified (Kendrick and Chang 2008, and refs herein); this protein binds to ETR1 and functions as a negative regulator of the down-stream ethylene signal, and it is also named Green-Ripe in tomato (Barry and Giovannoni 2007). As it is ethylene inducible, it was suggested to act in the negative feed-back.

With respect to transcription factors involved in ethylene signalling in several plants, EIN3 is a prevalent one. It acts downstream of the histidine kinase ethylene receptor, ETR1, and the Raf-like kinase (Kendrick and Chang 2008). In Arabidopsis, mutations at the EIN3 locus confer insensitivity to high level of exogenous and endogenous ethylene suggesting that EIN3 is required for ethylene responsiveness in both seedling and adult plant tissues (Chao *et al.* 1997). In addition, MADS-box transcription factors were also shown to respond to ethylene. Indeed, two tomato genes *TM29* and *LeMADSRIN* belonging to the SEPALATTA sub-family of the MADS box cluster family seem to play a role in tomato fruit development by also responding to ethylene (Barry and Giovannoni 2007). Similarly, its closest homolog in grape, previously known VvMADS4, but renamed VvSEP3, appears to be associated with berry ripening (Diaz-Riquelme *et al.* 2009, Boss *et al.* 2002).

Recently, some papers suggested the likely climacteric behaviour of some non-climacteric fruits. For instance, *Citrus* fruit exhibits autocatalytic ethylene synthesis under some experimental conditions. Indeed, exogenous ethylene was shown to modulate gene expression of *ert1* and *ers1* orthologs (Katz *et al.* 2004). In strawberries, another non-climacteric fruit, same orthologs were up-regulated by exogenous ethylene (Trainotti *et al.* 2004).

Binder (2008) stated that there is a "need to examine a variety of species and developmental processes to gain a full understanding of ethylene receptor function". As a first step, a sequence survey of these grape proteins, involved in ethylene signalling pathway, was performed to estimate the degree of similarity with known ethylene-proteins in other plant models. To verify a potential transcriptional control throughout berry development of these ethylene-genes and a correlation with ethylene accumulation occurring at week 7 after anthesis, we investigated transcript accumulation of those genes described above (*ert1*, *etr2, ers1, ein4, rte1, ctr1, ein3 and MADS-box4)* over two seasons by performing Real-Time PCR experiments at different stages of berry development in 'Cabernet Sauvignon'. Finally, the transcriptional response of these same genes was also monitored after addition to the berry cluster of exogenous ethylene and 1-MCP to determine whether or not these compounds might indirectly control the gene transcription.

Material and Methods

Plant material and gas treatments: 'Cabernet Sauvignon' grapevines, 30 year old, were grafted on 110 Richter rootstocks and grown in Toulouse, South-West of France, in a non-irrigated vineyard, Domaine de Candie. Full bloom occurred around mid-June. Five cluster samples, per treatment, were harvested weekly and stored at -80 °C until analysis. Each cluster was originated from a different vine. Ethylene or 1-methylcyclopropene (1-MCP), were applied at various times following full bloom, for a 24 h period, in a polyethylene bag wrapped around the cluster, at an initial concentration of 4 $\mu l \cdot l^{-1}$. Same conditions were applied to the control clusters. For these experiments, clusters growing in a shaded area of the vines were chosen to avoid direct effects due to sunlight exposure and overheating associated with such a treatment. After the 24 h periods of treatment, the clusters were sampled and stored at -80 °C until analysis; berries of each cluster were stored separately.

Gene biocomputing and phylogenetic studies: Different databases containing cDNA and protein sequences (NCBI (http://www.ncbi.nlm.nih.gov), GRAMENE (http://www.gramene.org/), DFCI Grape Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=grape)) were screened to identify the closest orthologs of the ethylene receptors in grapevine.

Phylogenetic trees were constructed from the ClustalW alignment using the Neighbor-Joining method by the MEGA program. ClustalW multiple sequence alignment was formed using the whole amino acids sequence and the default parameters of the MEGA package with the exception of the Protein Weight Matrix (BLOSUM) (Kumar *et al.* 2004). The scale bars per tree (0.1 and 0.05) represent the number of substitutions per site and the numbers next to the nodes are boostrap values from 1,000 replicates. The GenBank accession numbers of the ethylene-receptors and transcription factors are as follows: PcETR1 (AAL66191), MdETR1 (081122), FaETR1 (CAC48384), VvETR1 (AAF63755), AtETR1 (NP176808), CsETR1 (BAA85817), SlETR1 (AAB39386), LsETR1 (AAQ15122), PeETR1 (BAA37136), VvERS1 (XP_002272649), AtERS1 (NP18126), VrERS1 (AAD03598), SlERS1 (AAC49124), MdERS1 (AAM08931), PcERS1 (AAL66199), VvEIN4 (AM30288), AtEIN4 (NP187108), FaEIN4 (CAC48386), SlETR2 (AAU34078), AtETR2 (NP188956), LsETR2 (AF350322), VvETR2 (CAN84042), CsETR2 (BAA85819), MdETR2 (ABI58286), SlETR4 (AAU34076), SlETR6 (AAU34078), VvRTE1 (XP_002274106), AtRTE1 (NP_180177), SlRTE1 (ABD34616), VvCTR1 (CAO15968), MdCTR1 (ABI58288), AtCTR1 (NP195993), RhCTR1 (AAK40361), SlCTR1 (AAR89820), DmCTR1 (BAC80147), OsCTR1 (BAD25412), VvEIN3 (XP_00227638), AtEIN3 (NP_188713), CmEIN3 (AAK67355), SlEIL1 (AAK58857), OsEIN3 (AAZ78349), RhEIN3

(AAM20924), PpEIN3 (ABK35085), FsEIN3 (CAC09582), NtEIN3 (AAP03998).

RNA extraction and quantitative PCR: The RNA extraction steps were performed according to (El-Kereamy *et al.* 2003); one biological replicate was generated by grounding 10 berries taken randomly from the five cluster batches stored at -80°C. RNA extracts were transferred on mini-columns for DNase treatment with the Qiagen RNase free DNase kit, as specified by the manufacturer (Qiagen, Valencia, CA, USA). DNase-treated RNA (2 µg) was reverse transcribed in a total volume of 20 µl using Omniscript Reverse transcription Kit (Qiagen, Valencia, CA, USA).

Real-time PCRs were performed as previously described (Chervin *et al.* 2008) with some modifications. We used the equivalent of 5 ng of total RNA in a 10 µl total reaction volume per well. Quantitative PCR was performed using a set of specific primers given in Tab. 1. Grape gene sequences of orthologs to ethylene signalling receptors and transcription factors were found in DFCI Grape Gene Index (http://compbio.dfci.harvard.edu/tgi/plant.html), and the names were chosen to be similar to the Arabidopsis terminology. The prediction of orthology between these *Vitis vinifera* genes and other species orthologs is given on phylogenetic trees (Fig. 2). For all the genes studied here, optimal primer concentration was 300 nM. All RT-PCR experiments were run in triplicate with different cDNAs synthesised from three biological replicates. Each sample was run in three technical replicates on a 384-well plate. Relative fold differences (Transcript Accumulation Index: TAI) were calculated based on the comparative Ct method using the EF1-α as an internal standard and the $2^{-\Delta\Delta Ct}$ formula, with the highest ΔCt as the basal reference (the "one" value) for each gene in Fig. 1. In Fig. 2 the basal reference (the "one" value) was given to the controls. The EF1-α has been shown to be constitutively expressed along the grape berry development (Terrier *et al.* 2005). There were three to six biological replicates depending on the time.

Table 1

Quantitative PCR primers

Name	Oligonucleotide sequence
EF1-α (F)	5'-AACCAAAATATCCGGAGTAAAAGA-3'
EF1-α (R)	5'-GAACTGGGTGCTTGATAGGC-3'
ETR1 (F)	5'-AGAACACCTATGCATGCCATCA-3'
ETR1 (R)	5'-CTGCTCTTTAGGATGGCTTCAAC-3'
ERS1 (F)	5'-GCCCCTCACTTTCAATCCAA-3'
ERS1 (R)	5'-TGGACTCGCCATTTGTAAACG-3'
ETR2 (F)	5'-CCAAAAGCATGGCTCTCGTT-3'
ETR2 (R)	5'-TGGTTCAGAAATGTTGATTCCAA-3'
EIN4 (F)	5'-TTGAAGTAGCAAAAAGAATCCGAA-3'
EIN4 (R)	5'-GCTTGCTGTCAGGGCTATGAT-3'
RTE1 (F)	5'-GGTGGTTGGAATGTGGTGAAT-3'
RTE1 (R)	5'-ATCATAGATGTTGTGCTCACCCA-3'
CTR1 (F)	5'-GCACAAACCTGGTGCAAGAGA-3'
CTR1 (R)	5'-TCATGCCCTTGGCCACAT-3'
EIN3 (F)	5'-CTGGTGGGAGTGGATCTTTTG-3'
EIN3 (R)	5'-CCTATCTCTGGCTCCTACGCC-3'
MADS4 (F)	5'-AAATGCACAAGACGTGGGC-3'
MADS4 (R)	5'-GGATCGGGCTGGTATCCG-3'

Statistical analyses: Statistical significances were tested with ANOVAs using SigmaStat v3.0 (Systat Software, Inc., Point Richmond, CA); the LSD values were calculated at $P = 0.05$.

Results and Discussion

Some ethylene receptor genes of *Vitis vinifera*: structure and organization: A screening of different databases containing cDNA and protein sequences enabled us to identify four predicted mRNAs respectively named *VvETR1*, *VvETR2*, *VvERS1* and *VvEIN4* that might be the closest orthologs of the ethylene receptors in grapevine. The first one, *VvETR1* (Annotated Identifier by Genoscope: GSVIVT00000993001, http://www.genoscope.cns.fr/spip/), had a predicted length of 2229 bp and encoded a predicted open reading frame (ORF) of 742 amino acids protein. The comparison of the protein sequences via different protein databases revealed the presence of several putative conserved regions such as three hydrophobic regions within the amino terminal region, one GAF domain, Histidine ki-

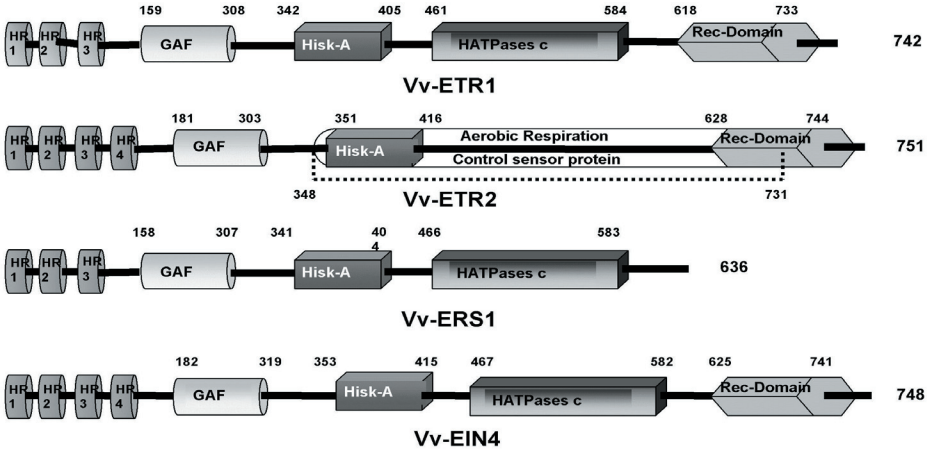

Fig. 1: Molecular organisation of the four ethylene receptors VvETR1, VvETR2, VvERS1 and VvEIN4. Check the Results and Discussion paragraph for the meanings and roles of the different domains.

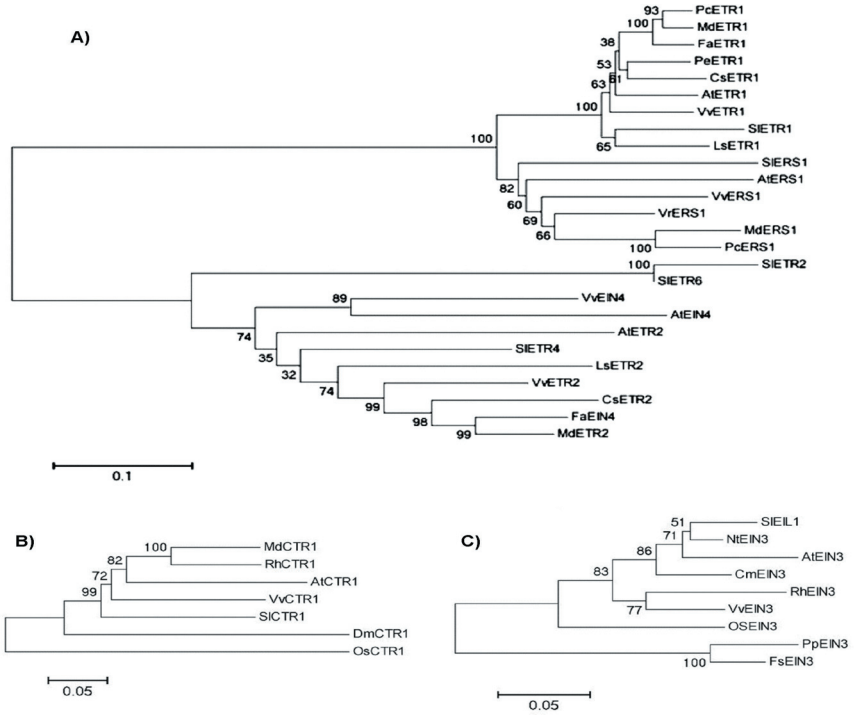

Fig. 2: Phylogenetic trees constructed from ClustalW alignments. **A**) Tree of ethylene receptors, orthologs to AtETR1, AtETR2, AtEIN4 and AtERS1; **B**) tree of the AtCTR1 orthologs, and **C**) tree of the orthologs of AtEIN3 transcription factor. The accession numbers are given in "Material and Methods". The scale bars per tree (0.1 and 0.05) represent the number of substitutions per site and the numbers next to the nodes are boostrap values from 1,000 replicates.

nase regions (HisKA, HATPases) and a receiver domain as already observed in tomato or in Arabidopsis thaliana (Figure 1). The ethylene binding domain is located at the N-terminal portion of the proteins where three or four hydrophobic transmembrane regions can be found (HR cylinders stands for hydrophobic region). The GAF domain is depicted as a clear cylinder and is part of a two-component system (GAF comes from GMP binding phosphodiesterase - Adenylate cyclase - Fh1A transcription factor). Likewise, two other domains can be depicted in the COOH-terminal portion: the Histidine Kinase A and the HATPase_c domains. The first one is a histidine kinase A domain, which is part of the two-component signalling system. This domain senses a signal input and transfer the signal through autophosphorylation to a response regulator protein. The second one is histidine kinase-like ATPases. This family includes several ATP-binding proteins such as histidine kinase, DNA gyrase B, topoisomerases. Then, the rhombus depicted a signal receiver domain identified in bacteria but also in eukaryotes such as ETR1 in Arabidopsis thaliana. This domain is supposed to receive the signal from the sensor partner in a two-component system. It contains a phosphoacceptor site that is phosphorylated by histidine kinase homologs. The aerobic respiration control sensor identified in VvETR2 does not belong to a superfamily domain but it is supposed to be part to the two component regulatory system in Archaebacteria. This Fig. 1 was adapted from Kendrick and Chang (2008), from Tanaka *et al.* (1998), and http://www.ncbi.nlm.nih.gov. Moreover, VvETR2 shared a strong similarity in protein sequence with two others putative ETR1 receptor identified in apple and in strawberry (Tab. 2). Likewise, its protein sequence shared 82 % of identity to AtETR1 suggesting potential similarities in protein function with the sub-family I of plant ethylene group.

VvETR2 protein sequence deduced from *VvETR2* gene (LOC100254638-NCBI Identifier; predicted length: 2809 bp) exhibited a predicted ORF of 764 amino acids with same putative domains as observed in VvETR1. Besides, it contained a large domain identified as a control sensor domain. The highest similarity in protein sequence for this predicted protein was observed with MdETR2 (*Malus domestica*) and FaEIN4 (*Fragaria ananassa*) with 76 % of amino acids identity while only 37 % of the amino acids are identical to SlETR2 from tomato, a climacteric fruit. On the other hand, VvETR2 protein sequence is close to that of SlETR4 in with 69 % of identity (Tab. 2) whose fruit-specific suppression in tomato causes early ripening (Kevany *et al.* 2008)

The third clone named *VvERS1* (GSVIVT00028044001; predicted length: 1911 bp) encoded a predicted ORF of 636 amino acids and shared strong homologies (62-74 % of identity) with most of the ERS1 proteins identified in tomato, apple or strawberry (Tab. 2). It contained all the domains identified in either VvETR1 or VvETR2, but lacks a receiver domain, like its homologs, in *Arabidopsis thaliana* (AtERS1) and tomato (Sl-ERS1), suggesting he might play a similar functional role in the ethylene signalling in grape.

Finally, the fourth clone named *VvEIN4* (GSVIVT0002180001; predicted length: 2283 bp) had a predicted ORF of 760 amino acids sharing significant homologies in protein sequence with members of EIN4 ethylene receptors sub-family (Tab. 2). Besides, it exhibits, like

Table 2

Amino acid sequence comparison between the predicted full length grape, Arabidopsis, tomato, apple and strawberry

	Protein size (no. of aa)	Amino acid identity (%)			
		VvETR1	VvETR2	VvERS1	VvEIN4
Vitis vinifera					
VvETR1	742	100			
VvETR2	751	37	100		
VvERS1	636	64	33	100	
VvEIN4	748	37	68	32	100
Arabidopsis thaliana					
AtETR1	738	82	36	63	36
AtERS1	613	57	31	72	30
AtETR2	773	37	61	31	55
AtEIN4	766	36	58	31	63
Solanum lycopersicum					
SlETR1	781	74	35	59	34
SlETR2	736	79	37	62	36
SlETR4	761	39	69	34	63
SlETR6	754	34	56	31	51
SlERS1	635	57	30	74	30
S1ERS4	761	38	68	62	61
Malus domestica					
MdETR1	741	85	37	65	37
MdERS1	605	55	28	73	28
MdERS1	765	37	76	33	66
Fragaria ananassa					
FaETR1	741	85	37	62	36
FaERS1	633	59	29	62	29
FaEIN4	765	37	76	62	67

VvETR2, an extra hydrophobic region within the amino terminal region. The highest homology in protein sequence was found with the EIN4 plants sub-family (61-67 %) supporting the assumption of a similar function in grape.

A phylogenetic analysis of the deduced protein sequences of these four receptors was compared to catalytically verified or putative protein of ethylene receptors from various plants (Fig. 2). The phylogenetic tree placed all these four receptors into respective clades that contained orthologs-related ethylene receptors regardless their classification as climacteric or non-climacteric fruit. Indeed, VvETR1 was placed into a sub-cluster containing ETR1 receptors from other plants and the most similar appeared to be AtETR1 with 82 % identity. On the other hand, VvERS1 and VvETR2 protein sequence appeared to be distinct within their sub-clusters (Fig. 2 A). Indeed, these two ethylene receptors were individually represented and did not belong to a specific sub-cluster. Finally, VvEIN4 was located to a sub-cluster containing an ethylene receptor of the sub-group II (AtETR2) suggesting a likely similarity in protein function between these members of ethylene sub-family receptors. As a conclusion, those four predicted proteins from grapevine can be legitimately classified as potential ethylene receptors.

A new "actor" in the ethylene perception, named RTE1 in Arabidopsis, has been characterised recently (Resnick *et al.* 2008, and refs herein). VvRTE1 shows reasonable similarities to Arabidopsis and tomato orthologs, 0.67 to AtRTE1 and 0.64 to SlRTE1, respectively; these values were obtained by optimal global alignment and similarity matrix Blosum62. But there were too few RTE1 proteins already characterised in different plant species to make a phylogenetic tree. It is not an ethylene receptor protein, and adding it in the receptor phylogenetic tree had no relevance. It is now suggested that RTE1 modifies the ETR1 receptor conformation, forcing it to an "ON" state, then to the "OFF" state of CTR1, which will blocks the downstream cascade (Resnick *et al.* 2008).

Additional phylogenetic trees have been built to compare VvCTR1 and VvEIN3 to plant orthologs (Fig. 2 B, C). It is not surprising to find genes for those two key elements in the ethylene signalling; indeed CTR1 transmits the signal from receptors to downstream transcription factors, like EIN3 (Barry and Giovannoni 2007, and refs herein). The phylogenetic tree of MADS boxes has been published recently (Diaz-Riquelme *et al.* 2009) a paper in which VvMADS4 according to Boss *et al.* (2002), is named VvSEP3.

The transcript accumulation of several ethylene-signalling receptors and transcription factors is developmentally regulated: Measurement of berry diameter and percentage of coloured berries over the entire development phase indicated that veraison, inception of ripening, occurred around week 7 to 8 after full bloom, with 50 % of the berries being coloured at week 9 (Fig. 3). A peak of internal ethylene was detected at week 7 before veraison (Chervin *et al.* 2004). Although the peak

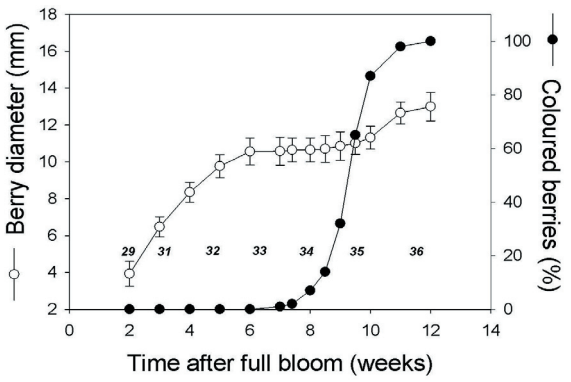

Fig. 3: Changes in berry diameter and coloured berries (% per cluster) of 'Cabernet Sauvignon' grapes as a function of the time after full bloom. The diameter was measured on batches of 50 berries, bars represent SD. The numbers in italics show E-L growth stages (Coombe 1995).

was obvious the quantities of internal ethylene were very small. This led us to check if an additional regulation could be the modulation of the ethylene receptors, and other receptors and transcription factors involved in the signalling cascade.

In Fig. 4, time course studies of genes encoding ethylene receptor or ethylene signalling proteins by Real Time PCR showed strong variations in transcript abundance depending on the gene. For instance, *VvETR2* mRNA accumulation (Fig. 4) peaked before veraison, concomitantly to the internal ethylene peak (pointed by the arrow) suggesting a likely correlation between the increase in gene expression of VvETR2 and the peak of ethylene production at week 7. Such variations in transcripts were observed in other plant model such as in strawberries prior to fruit ripening and in tomato during the mature green stage (Trainotti *et al.* 2005, Kevany *et al.* 2008 and 2007). In addition, these latter authors showed that the increase of such transcripts in tomato (*SlETR4* and *SlETR6*) is related to the degradation of the corresponding proteins and these variations between transcript and protein levels in tomato fruit might trigger the ripening process. It is worthwhile to note that VvETR2 is closely related to SlETR4 (Fig. 2, Tab. 2).

mRNAs encoding VvEIN4 and VvRTE1 accumulated steadily over the grape ripening phase (Fig. 4). Additional experiments will be needed to investigate their putative functions during the maturation phase in grape. In contrast, transcripts abundance of *VvCTR1* gene was nearly constant over the berry ripening despite of a slight peak at week 12, quite late in the ripening phase.

On the other hand, *VvEIN3* and *VvMADS4* transcript abundances showed a transient up-regulation at week 9 for *VvEIN3* and week 11 for *VvMADS4* (Fig. 4), which coincides with the maturation phase, colour changes (Fig. 3) and sugar accumulation (not shown here). Interestingly, *VvMADS4* transcripts accumulated later than *VvEIN3* during berry development. Given that the transcription of this gene may depend partly to ERFs (ethylene response factors), this suggests that ethylene signals are potentially still active at this stage.

Responses to exogenous ethylene and 1-MCP: To determine whether the receptor gene

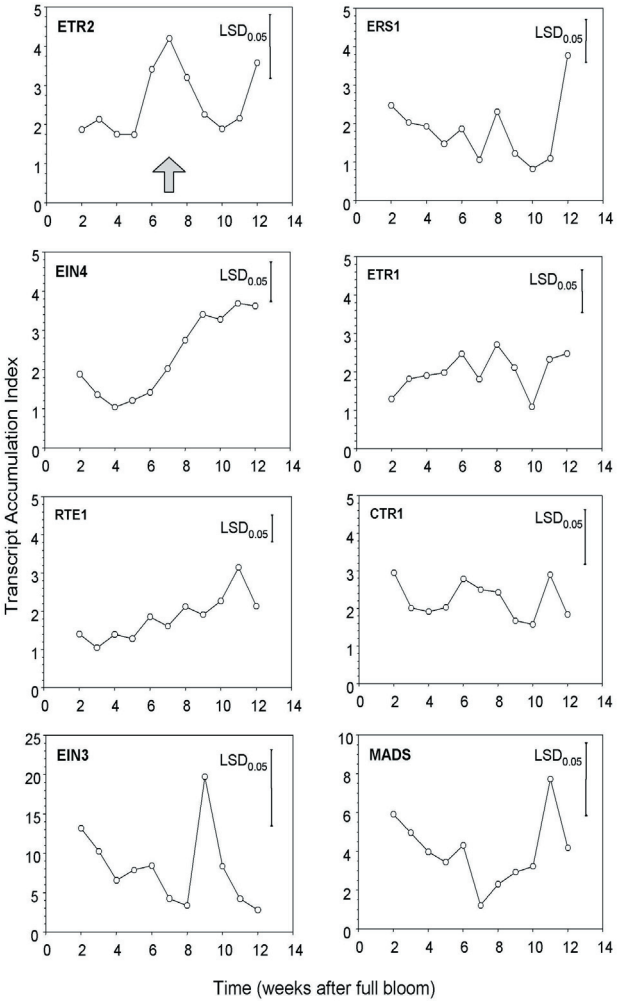

Fig. 4: Transcript accumulation of various ethylene receptor genes over berry development, the berries of 'Cabernet Sauvignon' grapes were collected in triplicates over two seasons, symbols are average of three to six biological replicates, LSD bars were calculated with ANOVAs over berry development. The arrow shows the approximate timing of the ethylene peak, as previously published (Chervin *et al.* 2004).

family is regulated by ethylene in grape berry, individual clusters were treated with ethylene and 1-MCP for 24 h at various stages of berry development. The application of exogenous ethylene led to approximately a 2-, 3-fold increase in *VvETR2* and *VvCTR1* transcripts at weeks 10 and 11 compared to the control (Fig. 5). This response generated by ethylene might correspond to the negative regulator function of these receptors: more ethylene would lead to more repressors of the signalling pathway. In tomatoes, application of exogenous ethylene led to a higher production of receptor transcripts (*SlETR4* and *SlETR6*), while their corresponding proteins were detected in smaller quantities (Kevany *et al.* 2007). Fine time-course experiments with Arabidopsis suggest that exogenous ethylene first increases receptor transcripts, then the corresponding protein is induced, but rapidly degraded when reaching a threshold level, while the transcripts still accumulate (Chen *et al.* 2007). Similar accumulations of transcript levels in response to ethylene have also been observed in non-climacteric fruits such as strawberries (Trainotti *et al.*

2005). Taken together, these results suggest the existence of common regulatory pathways in climacteric and non-climacteric fruit.

On the other hand, 1-MCP application results in a significant increase in *VvEIN3* gene transcription, and to a lesser extent the same trend is observed with *VvMADS4* gene, particularly at week 9 (Fig. 5). This last result indicates that a blockade of the ethylene receptors has a positive effect on *VvEIN3* transcript accumulation, one more feed-back in this complex ethylene signalling pathway. Further investigations would be necessary particularly with regard to VvEIN3 protein degradation. There were also some significant increases in *VvEIN3* transcripts after application of ethylene at weeks 5, 11 and 12, which may be part of complex feed-back processes. Finally, there was a noticeable increase of *VvRTE1* transcript at week 7 when 1-MCP was applied, but we did not observe strong variations in *VvEIN4* transcript accumulation following ethylene or 1-MCP pulses. Moreover, it is worthwhile to note that our sampling occurred 24h after treatment, thus the observed effect was sustained for a rather long period.

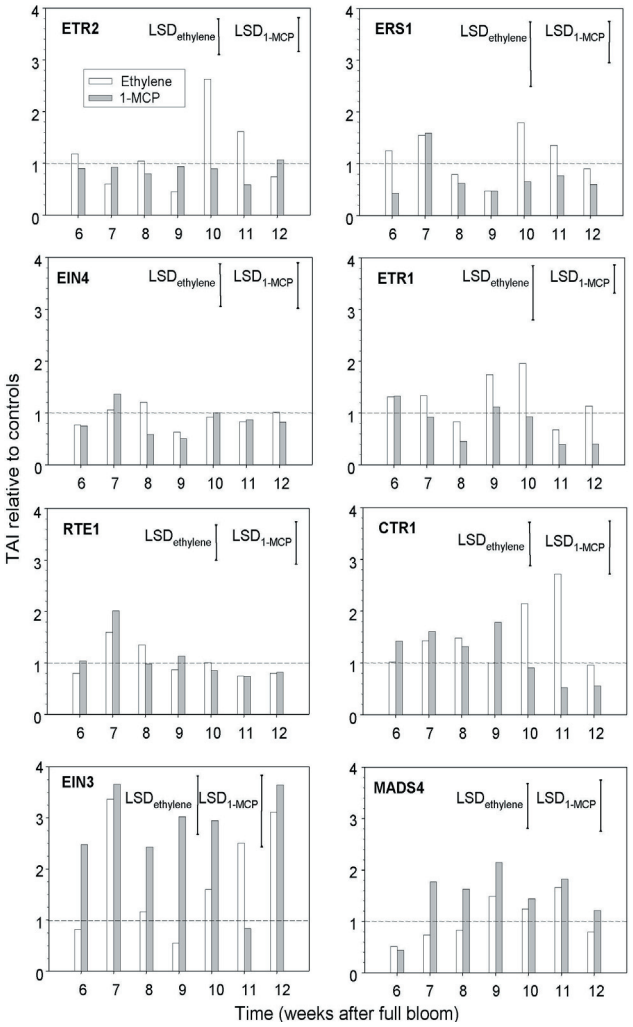

Fig. 5: Transcript accumulation of various ethylene receptor genes over berry development, after treatment with ethylene and 1-MCP for 24 h at an initial rate of 4 µl·l^{-1}, the control levels were given the "one" value; there were three biological replicates of 'Cabernet Sauvignon' berries, LSD bars were calculated with ANOVAs over berry development.

A role for ethylene signalling in the ripening process of grape berries? With respect to a likely role of ethylene in grape ripening, it would appear that the ripening of berries is triggered when some of these receptor transcripts are highly abundant while others exhibit a low abundance pattern. Whether some are prevalent in the inception of ripening and others are more critical to later events in the ripening process remains an open question. An in-depth investigation of the ethylene receptors at the protein level might help unravel the obscure role of ethylene in the grape berry ripening and might address this question: "Would a decrease of negative regulators (*i.e.* ethylene receptors) enhance the ripening process in absence of ethylene?"

Conclusion

Finally, this report describes the transcript abundance several ethylene receptors and transcription factors across berry development and the impact of one specific inhibitor of ethylene action on their respective mRNA accumulation. This study in grape berry tissues, a non-climacteric fruit, seems to indicate some similarities in the perception and the integration of the ethylene signalling with what was already observed in climacteric fruits. Moreover, our data suggest that key elements of the transcriptional signalling are developmentally regulated in grape berries. However, further experiments will be needed to extend this first set of data to the accumulation of the resulting proteins in order to better understand the mechanisms underlying the role of ethylene across berry ripening.

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